REMARKS

The present application relates to inbred maize plant and seed PH6WG. Claims 1-30 are pending in the present application. Claims 7, 9 and 19-22 have been amended. No new matter has been added by way of amendment. Applicants respectfully request consideration of the claims in view of the following remarks.

Detailed Action

Applicants acknowledge that the Terminal Disclaimer of June 15, 2005 has been reviewed and accepted and thus obviated the double patenting rejection of record.

Applicants further acknowledge the provisional rejection under § 101 double patenting of claims 5 and 6 has been withdrawn.

Finally, Applicants acknowledge that the Information Disclosure Statement (IDS) filed on January 29, 2004 has been considered and signed.

Double Patenting

Statutory Type Double Patenting

The Examiner rejects claim 11 under the statutory type double patenting under 35 U.S.C. § 101 as "claiming the same invention as that of claims 2 and 11 of the parent case, U.S. Patent No. 6,723,704". *See* Office Action, p. 2.

Applicants respectfully traverse this rejection. It is well established that Applicants have the right to claim the invention in a reasonable number of ways, and that a difference of scope between claims has been held to be enough. *See* MPEP § 706.03(k). Further, Applicants also point out that Claim 11 in the present continuation is based on Claim 6 in the case as originally filed. Claim 11 is not identical in scope to claims 2 and 11 of the parent case, U.S. Patent No. 6,723,704. Claim 11 of the present application claims "[a] maize plant having all the physiological and morphological characteristics of inbred line PH6WG, wherein a sample of the seed of inbred line PH6WG was deposited under ATCC Accession Number PTA-4530". In contrast claim 2 of U.S. Patent No. 6,723,704 claims "[a] maize plant, or a part thereof, produced by growing the seed of claim 1" and claim 11 claims "[a] method of producing an herbicide resistant maize plant comprising transforming the maize plant of claim 2 with a transgene that confers herbicide resistance".

Applicants believe the Examiner is making the assumption that the fact that one must use seed of the maize inbred line PH6WG itself to obtain a plant with the same morphological and physiological characteristics as a plant of the variety PH6WG. However, one of ordinary skill in the art can obtain a plant with all of the same morphological and physiological characteristics as maize inbred line PH6WG without actually using seed of maize inbred line PH6WG. For example, this can be accomplished by using double haploid technology to "recreate" PH6WG through the use of F1 hybrid seed in which PH6WG was a parent. As emphasized in previous office action responses, all members of the genus of F1 hybrids seed will receive one non-recombinant set of chromosomes of PH6WG. By using the seed of an F1 hybrid made with PH6WG, one can recover this non-recombined set of chromosomes from the F1 hybrid seed. Thus, a plant that has all of the same morphological and physical characteristics of PH6WG can be created without direct use of seed of inbred line PH6WG. Applicants direct the Examiner to the following web site which further explains and illustrates double haploid technology at the internet address www.uni-hohenheim.de/%7Eipspwww/350b/indexe.html#Project3 (attached as Appendix 1), as well as to U.S. Patent No. 5,770,788 to Jia and U.S. Patent No. 6,200,808 to Simmonds *et al.* As noted on the web site, the use of double haploid technology to has been used in plant breeding to produce desired homozygous inbred lines for more than 50 years.

Therefore, Applicants assert that claims 2 and 11 of U.S. Patent No. 6,723,704 are not duplicate claims, and requests reconsideration and withdrawal of the statutory type double patenting rejection under 35 U.S.C. § 101.

<u>Rejections Under 35 U.S.C. § 112, First Paragraph</u>

A. Written description regarding Claims 19-22

Claims 19-22 stand rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. The claim(s) contains subject matter, which was not described in the specification in such a way as reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner states that the "specification does not provide written description support for 'a single locus conversion' because the specification only describes a 'single gene conversion'". *See* Office Action, p. 9.

Although not acceding to the Examiner's rejection, in an effort to reduce the issues upon appeal, Applicants have now amended claims 19-22 to delete the language "locus" and include --gene--, as supported in the specification on page 21, thereby alleviating this rejection. Applicants further submit that the terms "single gene conversion" and "single locus conversion" are synonymous and would be well understood by one of ordinary skill in the art.

B. Enablement regarding Claims 1-10

Claims 1-10 remain and claims 13-16 and 19-29 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. The Examiner states the rejection is repeated for the reasons of record as set forth in the Office Action of March 15, 2005. *See* Office Action, pp. 9-10.

Applicants respectfully traverse. Applicants maintain the arguments submitted in previous amendment of June 15, 2005 regarding the references (Kevern, Carlone, and Segebart) mentioned by the Examiner.

Applicants further assert the specification provides a description of how to backcross traits into PH6WG (Specification, p. 21, ll. 16-31) and it is understood by those of skill in the art that backcross conversions are routinely produced and do not represent a substantial change to a variety. The World Seed Organization, on its web site, writes, "[t]he concept of an essentially derived variety was introduced into the 1991 Act of the UPOV Convention in order to avoid plagiarism through mutation, multiple back-crossing and to fill the gap between Plant Breeder's Rights and patents." ASSINSEL, an International breeders association, has published a position paper that refers to a conversion produced by repeated backcrossing of parental lines of hybrid varieties as a "cosmetic modification". As determined by the UPOV Convention, "essentially derived varieties may be obtained for example by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing, or transformation by genetic engineering" (emphasis added). Copies of web pages with these quotes are provided in Appendix 2. Thus, it is clear that there is worldwide agreement that by obtaining the seed of a newly developed variety such as PH6WG, and by using such seed for repeated backcrossing in accordance with claims 19-30, one is producing only a cosmetic modification and plagiarizing the work of the inbred inventor.

The ability of one of ordinary skill in the art to effectively use backcrossing to introgress a single locus conversion is also clearly supported by the scientific literature. For example, see Ragot, M. *et al.* (1995) Marker-assisted backcrossing: a practical example, in *Techniques et Utilisations des Marqueurs Moleculaires (Les Colloques*, Vol. 72, pp. 45-56 (attached as Appendix 3), and Openshaw *et al.*, (1994) Marker-assisted Selection in Backcross Breeding, Analysis of Molecular Marker Data, pp. 41-43 (attached as Appendix 4). Specifically, Ragot *et al.*, demonstrates that "spectacular" progress toward the recurrent parent genotype was obtained with 61 RFLP markers. Ragot *et al.* concludes that "recovery of the recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated."

Furthermore, the specification teaches multiple ways of introgressing or transforming a maize plant with various genes which encode specific protein products which confer advantageous traits desired in the plant. (*See* generally, specification, p. 23-34). This includes the use of markers to aid in the identification, selection and transformation of the maize plant with the desired gene.

Accordingly, Applicants submit that claims 1-10, 13-16, and 18-29 are fully enabled and have fully satisfied the legal standards for enablement. Applicants respectfully request reconsideration and withdrawal of the enablement rejections under 35 U.S.C. § 112, first paragraph.

Conclusion

In conclusion, Applicants submit in light of the above amendments and remarks, the claims as amended are in better condition for appeal. If it is felt that it would aid in prosecution, the Examiner is invited to contact the undersigned at the number indicated to discuss any outstanding issues.

No fees or extensions of time are believed to be due in connection with this amendment; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Respectfully submitted,

LILA A. T. AKRAD, Reg. No. 52,550
McKEE, VOORHEES & SEASE, P.L.C.
801 Grand Avenue, Suite 3200
Des Moines, Iowa 50309-2721
Phone No: (515) 288-3667
Fax No: (515) 288-1338
CUSTOMER NO: 27142

- LATA/bja -

Attorneys of Record

Application of the *in-vivo*-haploid induction in hybrid maize breeding

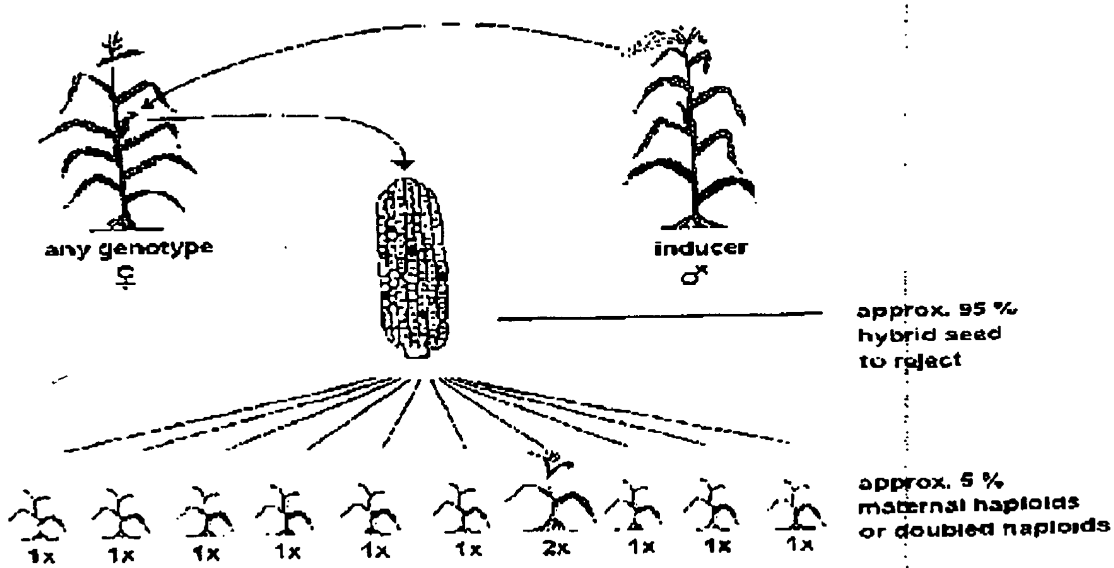
APPENDIX 1

1. Reproductive and genetic investigations on *in-vivo*-haploid induction in maize (*Zea mays* L.)

Contact person:
Prof. Dr. H.H. Geiger (geigerhh@uni-hohenheim.de)

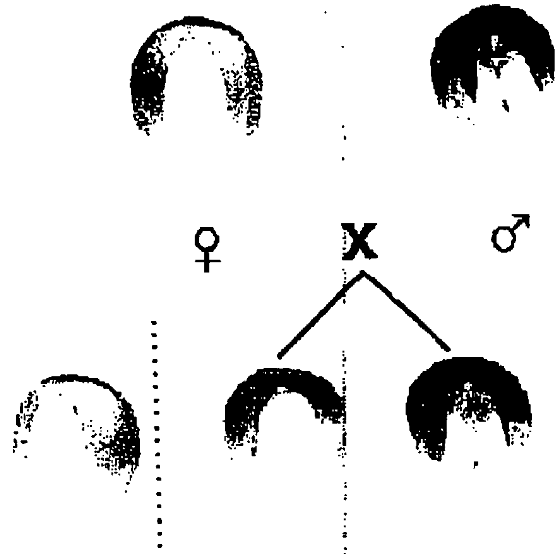
The interest in haploid/double haploid (H/D techniques has enormously increased in the years. The introduction of H/DH-techniques i maize breeding programs traces back to the 5 Shortly after the first reports of the spontane occurrence of H/DH-plants in maize, scientists a breeders started to discuss the application of su homozygous plants in breeding programs and th commercial use. By means of the developmen inductors and a method for artificial doubling of chromosome set, the H/DH-thechnique has be developed in the past years until such an extent it is beeing used as a matter of routine by ma breeders.

DH-Line in generation D_1



After pollination with an inducer plant, kernels with H-embryo of maternal origin with triploid endosperm arise, together with regularly

double fertilized kernels. Chromosome elimination and parthenogenesis are considered to be the possible biological mechanisms responsible for the occurrence of H-plants. However, chromosome elimination and parthenogenesis exclude each other per definition. Therefore, we chose the neutral term ***in-vivo*-haploid induction** for the phenomenon menitoned.



Inductor RWS

The aim of our work was to develop a novel indu line with an increased induction rate. The r **inducer line RWS** developed, displayes both advantage of a high induction rate and combination of two dominant identification mark a red stem, and an embryo and endosp coloration. Inducer RWS enables the breeder to *in-vivo*-haploid induction as an effective tool for development of H/DH-plants with almost genetic background. The method is less effec with donor genotypes, carrying the ab mentioned identification markers or anthozya inhibitor-genes themselves.

The spontaneous doubling rate in maize ran from 1-10 %. Therefore an artificial chromoso doubling method to increase the number of fe DH-plants is essential. The artificial chromoso doubling method, using colchicine as doubl agent, facilitates an effective development of lines.

Identification of H/DH-plants based on lacking stem-coloration

H/DH-field

ASSINSEL

Essential Derivation and Dependence

Practical Information

WHY THE CONCEPT OF ESSENTIAL DERIVATION?

The 1978 Act of the UPOV Convention (International Union for the Protection of New Varieties of Plants) states that "the authorization by the breeder shall not be required either for the utilization of the [his protected] variety as an initial source of variation for the purpose of creating other varieties or for the marketing of such varieties".

That principle, known as the "breeder's exemption", is essential for continued progress from plant breeding.

However, its implementation has progressively led to some abuses, due to the difficulties involved with assessment of distinctness, based on the text of the Convention (1978) which indicates that, for the basis of a title of protection, "the [new] variety must be clearly distinguishable by one or more important characteristics from any other variety whose existence is a matter of common knowledge ...".

Sometimes, "cosmetic modifications" were enough for protecting a new variety. That was particularly true in the case of mutation of ornamental or fruit plants and of "conversion" by repeated backcrossing of parental lines of hybrid varieties.

In order to improve the situation, in the early 1980's, a debate began on how to improve the system, trying to define "minimum distances" per species, but no consensus was reached. The development of genetic engineering, opened new possibilities for "piracy" of varieties and sped up the revision process of the Convention which, in the Act adopted in 1991, has introduced with the full agreement of breeders' associations, the concept of essential derivation. That concept of essential derivation has two aspects:

- a technical one: the question whether or not a plant variety is to be considered as a variety essentially derived from an initial variety;
- a juridical one: dependence, meaning that no protected acts as defined by the 1991 Act of the UPOV Convention (production, marketing ...) related to the essentially derived variety shall be carried out without the authorization of the owner of the protected initial variety.

DEFINITION OF AN ESSENTIALLY DERIVED VARIETY

The 1991 Act of the UPOV Convention states that "a variety shall be deemed to be essentially derived from another variety (the initial variety) when:

I. it is **predominantly derived** from the initial variety, or from a variety that is itself predominantly derived from the initial variety, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial

APPENDIX 2

variety;

ii. it is clearly distinguishable from the initial variety and

iii. except for the differences which result from the act of derivation, it conforms to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety.

Essentially derived varieties may be obtained, for example, by selection of natural or induced mutants, by selection of a somaclonal variant, by selection of variant individual plants in the initial variety, by backcrossing or by transformation (genetic engineering).

ASSINSEL interprets the definition given in the Convention as follows:

a) The technical aspects (matter of facts)

For a variety to be considered as essentially derived, it must fulfil three requirements in relation to the initial variety while retaining the expression of the essential characteristics of the initial variety:

i. clear distinctness in the sense of the UPOV Convention

ii. conformity to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety

iii. predominant derivation from an initial variety.

If one of these requirements is not fulfilled, there is no essential derivation.

The methods of breeding that can be regarded as leading to an essentially derived variety (see the above-mentioned explanatory list) may differ from species to species or even within a species. This may result in different thresholds being required to characterize essential derivation. Thus, conformity should be judged on a species-by-species or even within a species basis.

b) The juridical aspect

The principle of dependence only exists in favour of a protected variety. This means that:

i. the initial variety must be a protected one

ii. dependence can only exist from one protected variety alone

iii. an essentially derived variety can be directly derived from the initial variety or from a variety that is itself predominantly derived from the initial variety. It is possible to have a "cascade" of derivation. However, each essentially derived variety shall only be dependent on one, the protected initial variety. A cascade of dependence shall not exist, the principle having been introduced to better protect the breeder of the initial variety and not those having made derivations from his work.

ASSESSMENT OF ESSENTIAL DERIVATION

The assessment of essential derivation needs to take into account the three criteria mentioned above:

- clear distinctness in the sense of the UPOV Convention
- conformity to the initial variety in the expression of the essential characteristics that result from the genotype or the combination of genotypes of the initial variety
- predominant derivation from an initial variety.

The first criterion will be decided upon by the office in charge of granting a right to the breeder of the variety, according to the UPOV rule of distinctness.

The second criterion could be based on reliable phenotypic characteristics and/or on reliable molecular characteristics: either close relationship in general which could lead to a "conformity threshold" parallel to the minimum distance threshold used for distinctness or only small differences in some simply inherited characteristics. If this second criterion is considered as fulfilled, then, we have to assess the third one, which is "predominant derivation from an initial variety".

The third criterion, predominant derivation from an initial variety, implies that the initial variety or products essentially derived therefrom have been used in the breeding process.

In order to prove that use, various criteria or a combination thereof may be used:

- combining ability
- phenotypic characteristics
- molecular characteristics.

These criteria will have to be handled differently from their use for assessment of distinctness. Whatever solution retained, one will probably have to use distance coefficients to define thresholds. Up to now, ASSINSEL has essentially worked on thresholds based on distances measured by molecular markers. Geneticists and statisticians consider that technically it is equally possible to measure distance coefficients using phenotypic markers. However, the process would probably be more difficult due to environmental factors, and much more expensive: necessity of several testing locations during several years. However, if breeders prefer to use morphological markers instead of molecular markers, that should be possible.

The interest of using combining ability and the heterosis level will strongly depend on the crop. Thresholds will also be necessary.

The various ASSINSEL Sections are considering the establishment of thresholds for characterization of essential derivation according to this following general principle:

- One should propose, species by species, a first threshold below which a variety should be considered as non-essentially derived from an initial variety and a second threshold of conformity above which the new variety should be considered as essentially derived, except if the breeder can prove, by clear evidence, that he has started from independent germplasm.
- Between those two thresholds, the derivation could be disputable and the breeder of the putative essentially derived variety should have to give, in case of amicable negotiation or arbitration, information on the origin of the new variety. Should that information be unsatisfactory, the tribunal or of arbitrators/conciliators agreed on by both parties may request breeding records be provided for their examination.

This approach may be diagrammed as follows:

} Zone of non-derivation (Zone 1)

— Threshold No.1

} Zone of uncertainty (Zone 2)

— Threshold No.2

} Zone of non-distinctness or of indisputable derivation (Zone 3)

— 100%

Scale of genomic conformity

Some breeders are developing such scheme and call the zone No.1 "green zone", in which breeders would have freedom to operate. Zone No.3, the "red zone", where the breeder would know, according to his breeding materials, if his new variety is obviously essentially derived and dependent. Zone No.2 is where there would be uncertainty and where discussion may be appropriate. The threshold levels would be established first as an experiment. They could be further modified according to the experience acquired in the implementation of the scheme.

While this approach may be worthwhile, it also presents some obvious difficulties:

- Breeders have so far been unable to agree on threshold levels for any species;
- Even if the thresholds adopted by the industry had merit, they will not represent an absolute certainty and a court of law could pass judgment on other bases or guidelines.

Nevertheless, this approach does provide some framework in which breeders might proceed.

CONSEQUENCES FOR THE BREEDERS

The concepts of derivation and dependence do not, fortunately, abolish the "breeder's exemption" which is still stated in the 1991 Act. However, "cosmetic" improvement or plagiarism, which could sometimes have allowed the creation of distinct varieties in the sense of the UPOV Convention, will no longer allow the creation of independent varieties. The consequences for the breeders, the farmers and biological diversity more broadly should be positive and will certainly impact the breeder's work.

a) Choice of the parents

Breeders should be certain of their legal access and freedom to use all parent materials employed in their breeding programs. They would have to pay more attention to the results of their breeding work when working with protected varieties within the "breeder's exemption".

b) Breeding methods

Any conventional breeding method could, in theory, provide an essentially derived

variety. Certain methods appear to give a higher risk of developing essentially derived varieties. Among these methods we include:

- natural or induced mutations;
- repeated backcrosses; (discussions still continue on the number of backcrosses which could lead to an essentially derived variety. As shown in the French text of the 1991 Convention, which is of evidence, the authors of the Convention had in mind at least two backcrosses, the word being written in plural. However, it must be noted that the selection pressure exerted after the backcross(es) can have an important effect on the final result).
- selection in an existing variety, for example the choice of clones in a synthetic variety;
- transformation by genetic engineering.

c) Development of technical information

Conformity thresholds for essential derivation, such as presented above, can be defined in the frame of professional agreement (which would be the solution) or, in a case-by-case basis, in decisions by courts of law. In either case, thresholds willcome to exist in the years ahead. To know their freedom to operate in relation to such thresholds, breeders will need:

- a good knowledge of the range of phenotypic, molecular and physiological variability of varieties present in the market;
- to know the phenotypic, molecular and physiological profiles of their genetic material and their experimental varieties, as well as their breeding histories and documentation of legal access.

Breeders will need to employ the tools necessary for assessing such profiles in their research programs. Such tools will not only be used for the protection of Intellectual property, but should also promote improvement of breeding efficiency.

d) Keeping of breeding books

Conformity thresholds only, at least in the zone of uncertainty (orange zone), will not allow a decision on derivation and dependence. In case of litigation, information on parental material and breeding methods will be needed. Thus, breeders will need to maintain clear and accurate breeding records. We encourage breeders to seek competent professional legal advice on the best ways to develop and maintain these important records.

What is an "Essentially Derived Variety"?

The concept of essentially derived variety was introduced into the 1991 Act of the UPOV Convention in order to avoid plagiarism through mutation, multiple back-crossing and to fill the gap between Plant Breeder's Rights and patents, gap which was becoming important due to the development of the use of patented genetic traits in genetic engineering.

An essentially derived variety is a variety which is distinct and predominantly derived from a protected initial variety, while retaining the essential characteristics of that initial variety.

As indicated as an example in the UPOV Convention, essentially derived varieties may be obtained by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, back-crossing, or transformation by genetic engineering.

The commercialization of an essentially derived variety needs the authorization of the owner of the rights vested in the initial variety.

The concept of essentially derived variety does not at all abolish the Breeder's Exemption, as free access to protected plant varieties for breeding purposes is maintained. It is not a threat to biodiversity. On the contrary, it favors biodiversity, encouraging breeders developing and marketing original varieties.

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Techniques et utilisations des marqueurs moléculaires
Montpellier (France), 29-31 mars 1994
Ed. INRA, Paris 1996 (Les Colloques, n°72)

Marker-assisted backcrossing: a practical example

M. RAGOT[1], M. BIASIOLLI[1], M.F. DELBUT[2], A. DELL'ORCO[1], L. MALGARINI[1], P. THEVENIN[1], J. VERNOY[2], J. VIVANT[2], R. ZIMMERMANN[1] and G. GAY[1].

1 Ciba Seeds, CH-4002 Basle, Switzerland.

2 Département d'Amélioration des Plantes, INRA-Domaine d'Epoisses, F-21110 Genlis, France

Summary

That molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed. Restriction Fragment Length Polymorphisms (RFLP's) were used in maize to introgress by backcross a transgene construct, containing phosphinothricin resistance and insecticidal protein genes, from a transformed parent into an elite inbred line. At each generation plants carrying the transgene construct were selected based on their phosphinothricin resistance, and further characterized with RFLP's. Both maximum recovery of recurrent parent genotype and minimum linkage drag were taken into account for marker-based selection. Embryo rescue was used to shorten generation time. Progress towards recurrent parent genotype was spectacular. Levels of recurrent parent genotype recovery which would normally be observed, in the absence of selection, in the BC_6 generation were obtained at the BC_3 generation, about one year after BC_1 seeds had been planted. Besides the evidence already provided by RFLP's, phenotypic evaluation of the backcross-derived near-isogenic lines will constitute an additional check of the completeness of the conversion.

Introduction

Backcrossing has been a common breeding practice for as long as elite germplasm has been available. It has mainly been used to introgress single Mendelian traits, such as disease resistances or quality factors, into elite germplasm (Allard 1960; Hallauer and Miranda 1981). One of the most attractive attributes of backcrossing is that it allows to perform targeted modifications without disrupting the existing overall genetic balance of the recurrent parent.

However, production of fully converted near isogenic lines through classical backcrossing procedures is a lengthy procedure, if at all possible. Theoretically, a minimum

APPENDIX 3

of seven classical backcross generations are required to recover more than 99% of recurrent parent genotype, assuming no linkage drag. The attractiveness of classical backcross procedures is therefore substantially diminished for crops, such as maize (*Zea mays* L.), where the turn-over of elite cultivars is very fast. In addition, full recovery of recurrent parent genotype is usually not achieved through classical backcrossing, which may result in deleterious agronomic effects. Murray *et al.* (1988) reported about 90% recurrent parent genotype recovery in two BC_{10}-equivalent conversions (A632Ht and A632Rp) of the maize line A632. The conversions had retained respectively 4 and 7 donor fragments in addition to the one carrying the gene of interest.

Reduction in the number of backcross generations needed to obtain fully converted individuals has been shown theoretically, or from simulations, to be achievable through the use of molecular markers (Tanksley *et al.* 1989; Hospital *et al.* 1992; Jarboe *et al.* 1994). Because they provide thorough characterization of the genetic variability at each backcross generation, markers allow to take full advantage of this variability by applying the highest possible selection intensity.

Efficiency of marker-assisted backcrossing was investigated through an experiment aimed at introgressing a single genetic factor (a transgenic construct) from a donor into a recipient maize line.

Materials and methods

Plant Material

A hemizygous transgenic maize line of Lancaster origin was used as donor parent to introgress its transgene construct, through repeated backcrossing, into a recipient parent from the Stiff Stalk germplasm group. Both parents are proprietary elite lines. The transgene construct carries both a phosphinothricin resistance gene and synthetic genes encoding the entomotoxic fragment of the CryIA(b) *Bacillus thuringiensis* protein (Koziel *et al.* 1993). Transformation was achieved through microprojectile bombardment (Koziel *et al.* 1993) and resulted in a single insertion (*Bt* locus), on chromosome 1 (Figure 1).

Backcross protocol

The F1 progeny of the cross between the donor and the recipient was screened for the presence of the transgene construct by applying Basta, a phosphinothricin-based herbicide, onto each plant. Resistant individuals were then used to generate BC_1 progeny.

For each backcross generation, except the BC_4, individuals were planted in multipots and sprayed with Basta to eliminate those which did not carry the transgene construct. To avoid the stress resulting from treatment with Basta, BC_4 plants carrying the transgene construct were identified using Southern blots probed with the *pat* and *Bt* genes. Resistant plants were transplanted in an open-soil greenhouse and leaf-sampled for molecular marker

analyses. Results of marker an
flowering. A single plant was
rescued and transferred onto t
embryos first underwent a gr
culture medium, before being
average, four months.

Molecular marker analys

Restriction Fragment Le
genotypes in all four genera
chemiluminescent techniques. 1
were chosen from among those
provided coverage of the entire
contained two loci tightly linked
recombination units away (Figu
BC_{n+1} generation comprised bo
or tightly linked ones, and addi
selected BC_n plant was heterozy
independent reference populati
generation.

Selection procedure

At each generation plants
recurrent-parent-genotype and
attempt to integrate both crite
missing values were not include
contributed to the selection proc
best ranking one of those for w
for the BC_3 selection) was avail

Results and discussion

Selection for the gene o

The observed segregation
significantly different ($P<0.05$

Recurrent parent genoty

Statistics for the genotyp
performed taking the whole g
backcross-derived plant there

recover more than 99% of recurrent
tractiveness of classical backcross
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(A632Ht and A632Rp) of the maize
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l *et al.* 1992; Jarboe *et al.* 1994).
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ne construct) from a donor into a

origin was used as donor parent to
ckcrossing, into a recipient parent
are proprietary elite lines. The
sistance gene and synthetic genes
lus thuringiensis protein (Koziel *et*
rojectile bombardment (Koziel *et*
chromosome 1 (Figure 1).

the recipient was screened for the
phosphinothricin-based herbicide,
nerate BC_1 progeny.
dividuals were planted in multipots
carry the transgene construct. To
BC_4 plants carrying the transgene
th the *pat* and *Bt* genes. Resistant
leaf-sampled for molecular marker

analyses. Results of marker analyses were made available at the latest two weeks after flowering. A single plant was selected, of which all backcross-derived embryos were rescued and transferred onto tissue culture medium. Plantlets that developed from these embryos first underwent a greenhouse acclimation phase, while still growing on tissue culture medium, before being transplanted into multipots. Backcross cycles lasted, on average, four months.

Molecular marker analyses

Restriction Fragment Length Polymorphisms (RFLP's) were used to establish genotypes in all four generations. RFLP detection involved either radioactive or chemiluminescent techniques. For the BC_1 generation, 61 marker-enzyme combinations were chosen from among those revealing polymorphism between donor and recipient. They provided coverage of the entire genome, defining intervals of about 25 cM in size, and contained two loci tightly linked to the *Bt* locus, CG320 and CG415, respectively 5 and 16 recombination units away (Figure 1). For subsequent generations, markers analyzed in the BC_{n+1} generation comprised both those for which the selected BC_n plant was heterozygous, or tightly linked ones, and additional ones located in chromosomal segments for which the selected BC_n plant was heterozygous (Table 1). Marker map positions were obtained from independent reference populations and confirmed by analysis of segregation in the BC_1 generation.

Selection procedure

At each generation plants were ranked based both on the percentage of homozygous recurrent-parent-genotype and on the extent of linkage drag around the *Bt* locus, in an attempt to integrate both criteria. Plants for which two or more adjacent markers had missing values were not included in the analyses. Success or failure of the pollinations also contributed to the selection procedure. One single plant was selected at each generation: the best ranking one of those for which a backcross progeny of size 100 or more (50 or more for the BC_3 selection) was available.

Results and discussion

Selection for the gene of interest

The observed segregation ratios for phosphinothricin resistance (Table 1) were not significantly different ($P<0.05$) from the expected 1:1, as shown by Chi-square tests.

Recurrent parent genotype recovery

Statistics for the genotyped plants are summarized in Table 1. Calculations were performed taking the whole genome into account, including the *Bt* locus. The "perfect" backcross-derived plant therefore counts one heterozygous chromosome segment, that

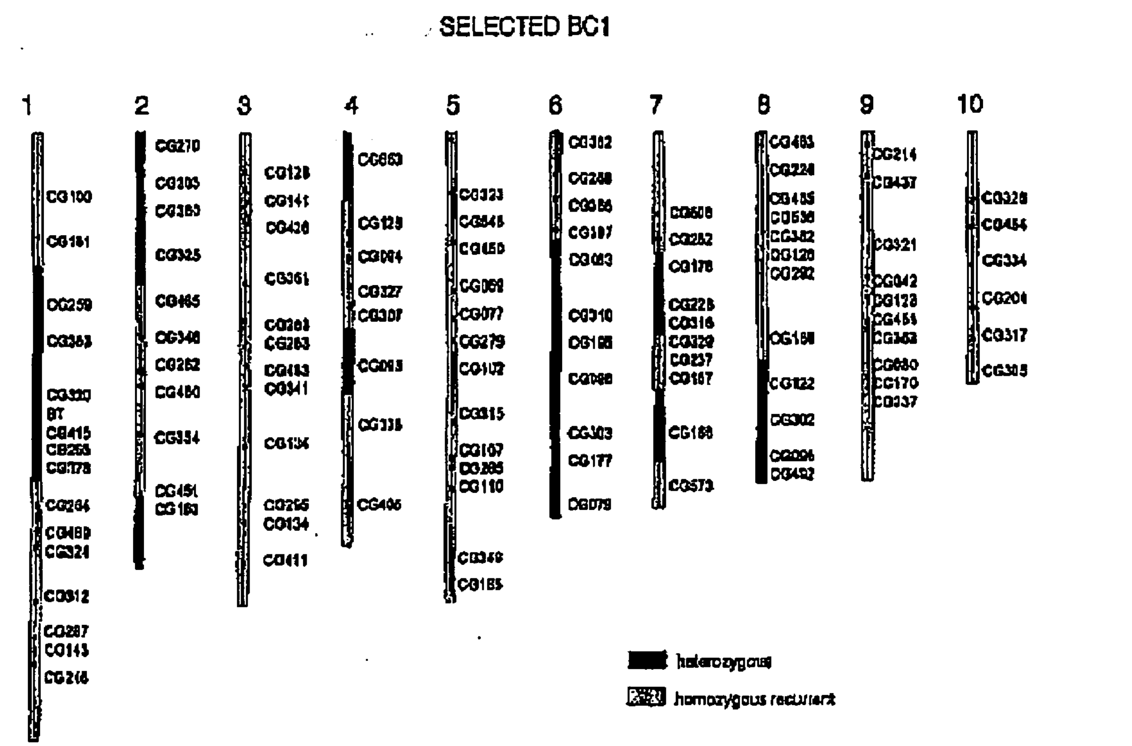
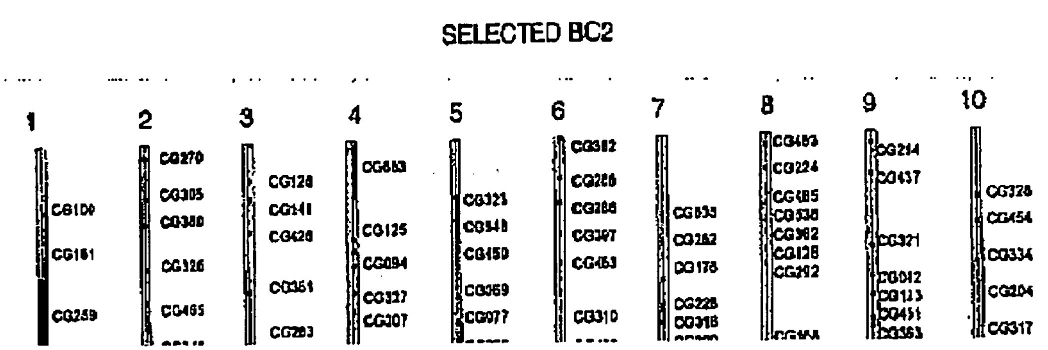


Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bi*) is located on chromosome 1.



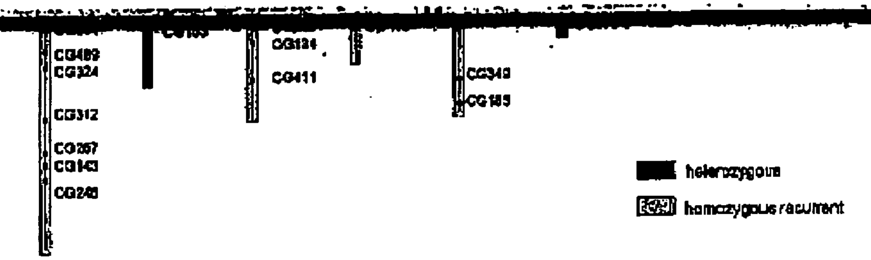


Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

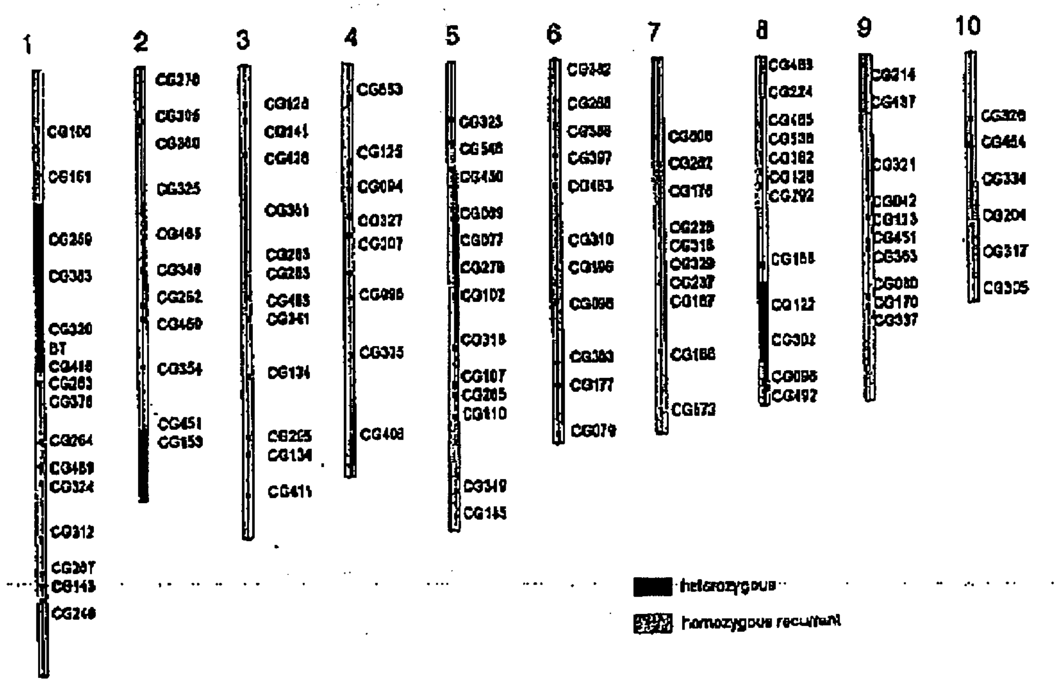


Figure 1-b: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

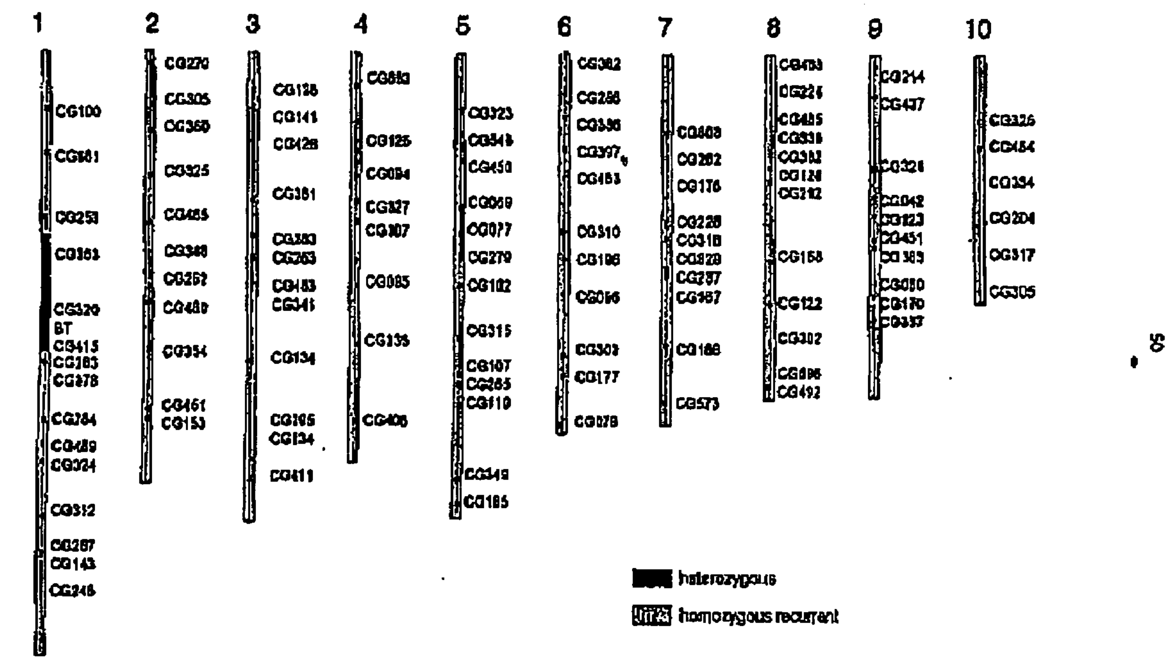
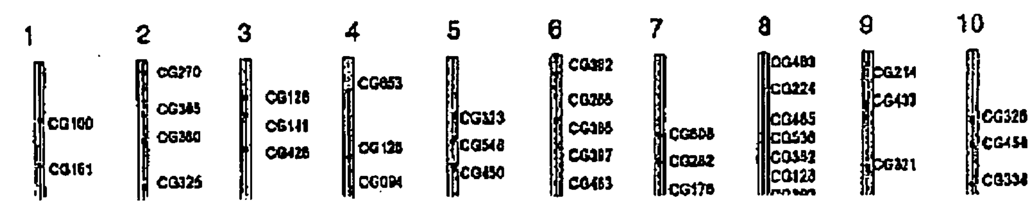


Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.



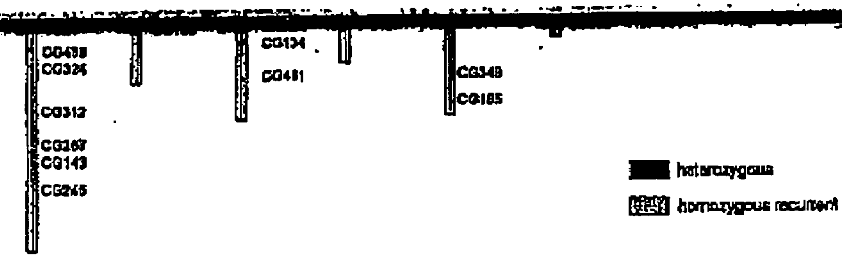


Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

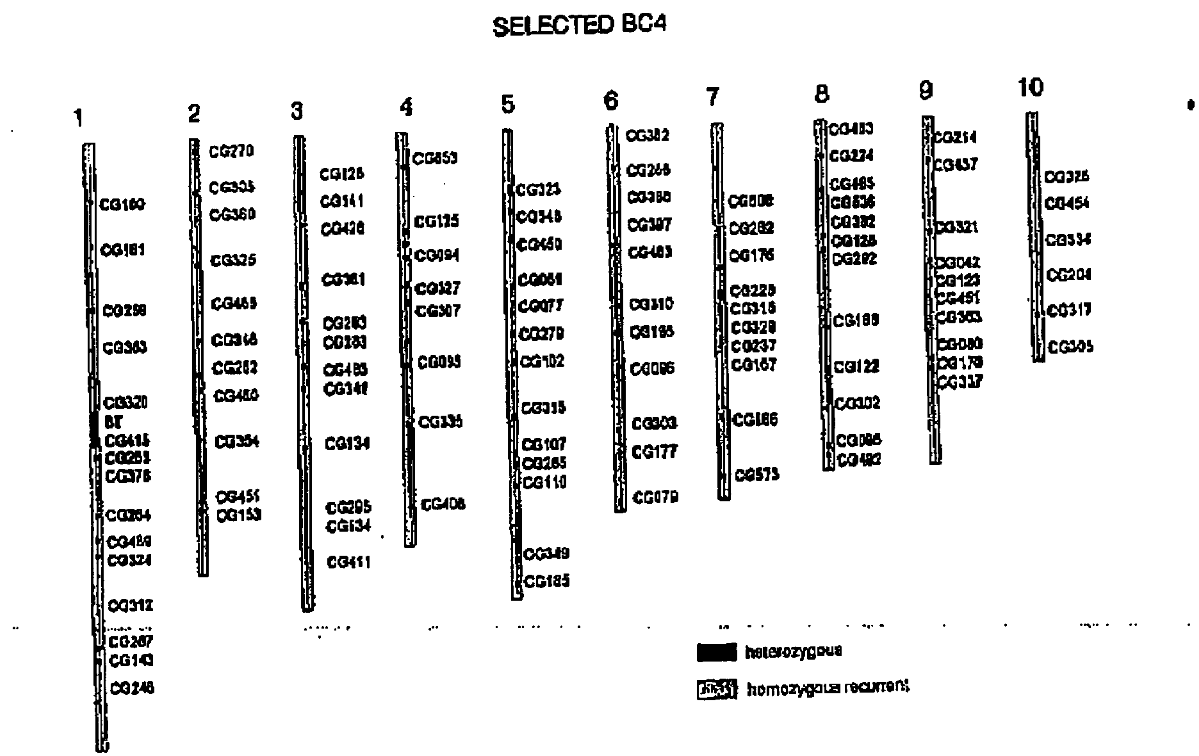


Figure 1-d: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

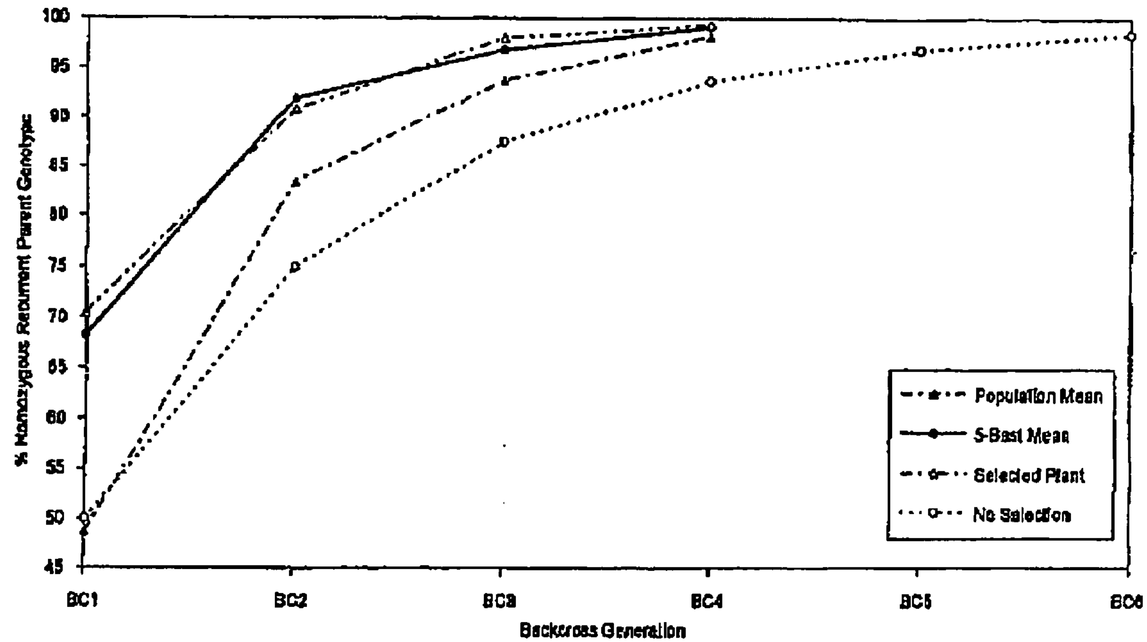


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin	RFLP genotyping	nb plants	% homozygous recurrent	nb heterozygous

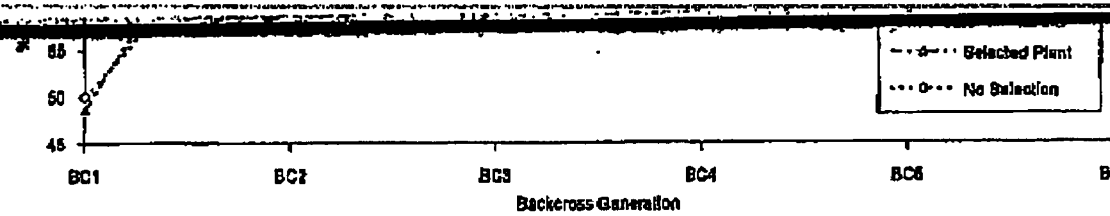


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin resistant plants	RFLP genotyping			nb plants analyzed *	% homozygous recurrent parent genotype				nb heterozygous chromosome segments ***			
		nb plants	nb loci	nb datapoints		mean	std dev	5-best mean **	selected plant	mean	std dev	5-best mean **	selected plant
BC1	49.05	98	61	5866	87	48.72	10.35	68.31	70.45	11.01	2.17	7.75	6
BC2	44.65	61	22	1342	30	83.42	5.64	91.98	90.84	5.03	1.54	3.20	3
BC3	46.32	72	10	720	71	93.83	1.85	96.82	98.03	2.20	0.71	1.60	1
BC4	-	26	3	78	28	98.23	0.49	99.09	99.36	1.00	0.00	1.00	1

* Plants for which two or more adjacent markers had missing values were not included in the analyses

** Mean value of the five individuals having the five highest percentages of homozygous recurrent parent genotype.

*** Including the segment carrying the transgene construct.

comprising the *Bt* locus. It also displays 99.36% of homozygous recurrent-parent-genotype. The remaining 0.64% corresponds to the average relative length of the chromosome segment containing the *Bt* locus, which depends on the two flanking markers chosen.

The mean percentage of homozygous recurrent-parent-genotype of the BC_1 generation was slightly lower than the expected 50%. This can be explained by linkage drag around the *Bt* locus, given that this percentage was computed based only on plants selected for heterozygosity at the *Bt* locus. For all other backcross generations the mean percentage of homozygous recurrent-parent-genotype was much higher than what would have been observed, should no selection have been done (Figure 2).

The percentage of homozygous recurrent-parent-genotype of the selected plant (Table 1) and the average of the five largest values (Table 1) were always very similar to one another, and much superior to the population mean value (Figure 2). The percentage of homozygous recurrent-parent-genotype of the selected plant was found only once, in the BC_2 generation, to be smaller than the average of the five largest values. This corresponded to the only time when the selected plant was not the one with the maximum percentage of homozygous recurrent-parent-genotype. The plant had been selected because it displayed a favorable recombination on one side of the *Bt* locus (Figure 1).

The percentage of homozygous recurrent-parent-genotype of the selected BC_1 plant was almost equal to that of an unselected BC_2, that of the selected BC_2 was larger than that of an unselected BC_3, that of the selected BC_3 was barely smaller than that of an unselected BC_6, and that of the selected BC_4 was equal to that of the "perfect" backcross-derived plant, given the set of markers that was used. Such rates of recurrent parent genotype recovery are consistent with results of simulation analyses. Jarboe *et al.* (1994) who used the maize genome as a model reported that three backcross generations and 80 markers were needed to recover 99% of recurrent parent genotype.

Number of donor chromosome segments

The number of heterozygous chromosomal segments decreased from one backcross generation to the next. Plants selected at each generation were not necessarily those which had the lowest number of heterozygous chromosomal segments (Table 1). However, with the set of markers used, BC_3 and BC_4 plants were recovered which contained only one heterozygous chromosomal segment: that comprising the *Bt* locus.

Linkage drag

Linkage drag around the *Bt* locus was estimated, relative to the length of chromosome 1. Its value was found to lie between 24.0 and 48.4% for the selected BC_1 individual, between 17.6 and 34.8% for the selected BC_2, between 2.0 and 24.0% for the selected BC_3, and between 0.0 and 8.4% (respectively 0.0 and 14.5 cM) for the selected BC_4.

The two values given for each ge
correspond to extreme positions o
flanking the transgene construct locu
BC_4 is likely to be less than 1.3%
appear to be somewhat high, reflec
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Tanksley (1989) found that the sizes
cM.

Conclusion

These results clearly demonstr
quality advantages over classical
through backcrossing. Only four ba
than a year and a half from plan
genotypically fully converted. Neve
genotype could proceed even faste
appropriate protocol and resources
allocated.

Comparison of BC_4-derived l
markers and agronomic performanc
order to confirm the completeness o

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14.5 cM) for the selected BC_4.

The two values given for each generation are extreme values of linkage drag, which correspond to extreme positions of the crossing-overs in the marker-defined intervals flanking the transgene construct locus. Therefore the true linkage drag value of the selected BC_4 is likely to be less than 1.3% of the genome. Although this maximum value may appear to be somewhat high, reflecting the limited selection pressure put here on linkage drag, it is much lower than what would be expected from classical backcross programs (Stam and Zeven 1981; Tanksley *et al.* 1989). Practically, in a study of *Tm*-2 conversions of tomato cultivars obtained by a large number of classical backcross cycles, Young and Tanksley (1989) found that the sizes of the introgressed fragments ranged between 4 and 51 cM.

Conclusion

These results clearly demonstrate that molecular markers provide important time and quality advantages over classical procedures for the production of near-isogenic lines through backcrossing. Only four backcross generations were necessary to recover, in less than a year and a half from planting of the BC_1's, individuals which appeared to be genotypically fully converted. Nevertheless, it is likely that recovery of recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated.

Comparison of BC_4-derived lines with the recurrent parent for both morphological markers and agronomic performance (including hybrid performance) will be performed in order to confirm the completeness of the conversion.

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Marker-assisted Selection in Backcross Breeding

S.J. Openshaw

Pioneer Hi-Bred Intl. Inc., P.O. Box 1004, Johnston, IA 50131

S.G. Jarboe[1]

CIMMYT, Lisboa 27, Apdo. Postal 6-641, 06600 Mexico, D.F., Mexico

W.D. Beavis

Pioneer Hi-Bred Intl. Inc., P.O. Box 1004, Johnston, IA 50131

Abstract. **The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Genetic markers can increase the effectiveness of backcrossing by 1) increasing the probability of obtaining a suitable conversion, and 2) decreasing the time required to achieve an acceptable recovery. Simulation and field results indicated that, for a genome consisting of ten 200-cM chromosomes, basing selection on 40 or 80 markers in 50 BC individuals that carry the allele being transferred can reduce the number of backcross generations needed from about seven to three.**

The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Usually, the trait being transferred is controlled by a single gene, but highly heritable traits that are more complexly inherited have also been transferred successfully by backcrossing; for example, maturity in maize (Rinke and Sentz, 1961; Shaver, 1976). Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm.

Several plant breeding textbooks give good descriptions of the backcross procedure (Allard, 1960; Fehr, 1987). A donor parent (DP) carrying a trait of interest is crossed to the recurrent parent (RP), an elite line that is lacking the trait. The F_1 is crossed back to the RP to produce the BC_1 generation. In the BC_1 and subsequent backcross generations, selected individuals carrying the gene being transferred are backcrossed to the RP. The expected proportion of DP genome is reduced by half with each generation of backcrossing. Ignoring effects of linkage to the selected DP allele being transferred, the percentage recurrent parent (%RP) genome expected in each backcross generation is calculated as:

$$\%RP = 100\,[1-(0.5)^{n+1}]$$

where n is the number of backcrosses.

Backcrossing of selected plants to the RP can be repeated each cycle until a line is obtained that is essentially a version of the RP that includes the introgressed allele. After six backcrosses, the expected recovery is >99% (Table 1).

Until recently, discussions of the recovery of the RP genome during backcrossing have emphasized the expected values for %RP shown in Table 1, and have largely ignored the genetic variation for %RP that exists around the expected mean. With the development of genetic markers capable of providing good genome coverage, there has been interest in taking advantage of that variation to increase the efficiency of backcrossing.

Selection for RP marker alleles can increase greatly the effectiveness of backcross programs by allowing the breeder to 1) select backcross plants that have a higher proportion of RP genome, and 2) select backcross individuals that are better conversions near a mapped donor allele being transferred (i.e., select for less linkage drag). Expressed in practical terms, using genetic markers to assist backcrossing can 1) increase the probability of obtaining a suitable conversion, and 2) decrease the time required to achieve an acceptable recovery.

Issues to consider when planning a marker-assisted backcross program include 1) the time advantage of using markers to assist backcrossing, 2) the number of markers needed, and 3) the number of genotypes to evaluate. In this report, we use results from previous literature, computer simulation, and empirical studies to provide some guidelines.

Table 1. *Expected recovery of recurrent parent (RP) genome during backcrossing, assuming no linkage to the gene being transferred.*

Generation	%RP
F_1	50.0000
BC_1	75.0000
BC_2	87.5000
BC_3	93.7500
BC_4	96.8750
BC_5	98.4375
BC_6	99.2188
BC_7	99.6094

[1] Formerly with Purdue University, West Lafayette, Ind.

APPENDIX 4

Materials and methods

The maize genome was the model for the simulation. The simulated genome contained ten 200-cM chromosomes. Simulation of crossing over was based on a Poisson distribution with a mean of 2.0 ($\lambda = 2$) (Hanson, 1959), which, on average, generated one cross over for every 100-cM length. The simulations reported here assume no interference. Codominant genetic markers were evenly distributed in the genome and sites of the donor gene were randomly assigned to genome locations.

Simulations were conducted with the following parameters:

Number of progeny: 100 or 500.
Backcross generations: BC_1, BC_2, and BC_3.
Number of markers: 20, 40, 80, or 100.
Number selected to form the next BC generation: 1 or 5.

Selection was based on 1) presence of the donor allele and 2) high %RP). %RP was calculated as the average of the (one or five) selected individuals. Values presented are the mean of 50 simulations.

Results

In the computer simulation study, all methods modeled greatly increased the speed of recovering the RP genome compared to the expected recovery with no marker-assisted selection (compare Tables 1 and 2). At least 80 markers were required to recover 99% of the RP genome in just three BC generations (Table 2). Use of at least 80 markers and 500 progeny allowed recovery of 98% RP in just two BC generations. Response to selection was diminished only slightly by spreading the effort over five selections. Using markers, the number of backcross generations needed to convert an inbred is reduced from about seven to three.

By the BC_3 generation, there appears to be no practical advantage to using 500 vs. 100 individuals. If the presence of the donor trait in the backcross individuals can be ascertained before markers are genotyped, then only half the number of individuals indicated in the tables will need to be analyzed.

When a small number of markers are used, they quickly became non-informative; i.e., selection causes the marker loci to become fixed for the RP type before the rest of the genome is fully converted (Table 3; Hospital et al., 1992). This situation was most prominent in the larger populations, where a higher selection intensity placed more selection pressure upon the marker loci. Accordingly, it is of interest to consider how closely the estimation of %RP based on markers reflects the actual genome composition. The combination of estimation of %RP based on fewer markers and subsequent selection tends to bias the estimates upward (compare Tables 2 and 3).

The results from the simulation compare well with real field data. In a typical example, 50 BC_1 plants carrying the gene being transferred were genotyped at 83 polymorphic RFLP loci (note that this corresponds to a population size of 100 unselected plants in Tables 2 and 3). The five best BC_1 recoveries had estimated %RP values of 85.9%, 82.7%, 82.0%, 81.4%, and 81.2%. After evaluating 10 BC_2 plants from each selected BC_1, the best BC_2 recovery had an estimated %RP of 94.6%.

Discussion

The simulations (Table 2; Hospital et al., 1992) and our experience indicate that four markers per 200-cM chromosome is adequate to greatly increase the effectiveness of selection in the BC_1. However, using only four markers per 200 cM will likely make it very difficult to map the location of the gene of interest. Adequate summarization of the data is an important

Table 2. *Percent recurrent parent genome during marker-assisted backcrossing.*

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_1	84.5	84.5	84.2	88.0	89.9	90.7	90.2	90.5
BC_2	95.0	95.2	95.8	97.2	96.5	97.7	98.5	98.6
BC_3	97.4	97.6	98.9	99.2	97.7	98.3	99.4	99.5
				Five selected				
BC_1	82.9	85.1	84.9	84.7	87.7	88.1	88.9	88.9
BC_2	93.7	95.0	95.8	95.7	95.5	96.8	97.8	97.9
BC_3	97.1	98.3	98.8	98.9	97.3	98.5	99.3	99.3

Table 3. *Estimates of percent recurrent parent genome, based on marker loci.*

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_2	98.7	97.8	95.6	97.2	100.0	99.1	98.6	98.0
BC_3	100.0	99.6	99.3	99.5	100.0	100.0	99.9	98.2
				Five selected				
BC_2	96.4	96.5	96.2	95.8	100.0	98.5	98.3	98.2
BC_3	99.9	99.8	99.3	99.1	100.0	100.0	99.9	99.8

part of a marker-assisted backcross program. Ideally, the markers used can supply data that can be represented as alleles of loci with known map position. Estimation of %RP, mapping the position of the locus of interest, and graphical display of the results (Young and Tanksley, 1989) are all useful in understanding and controlling the specific backcross experiment being conducted.

It appears that, with the use of genetic markers, the portion of the RP genome that is not linked to the allele being transferred can be recovered quickly and with confidence. The recovery of RP will be slower on the chromosome carrying the gene of interest. A considerable amount of linkage drag is expected to accompany selection for the DP allele in a backcross program. For a locus located in the middle of a 200-cM chromosome, the length of the DP chromosome segment accompanying selection is expected to be 126, 63, and 28 cM in the BC_1, BC_2, and BC_3 generations, respectively (Hanson, 1959; Naveira and Barbadilla, 1992). Our observations support the recommendation of Hospital et al. (1992) that preference be given to the selection for recombinants proximal to the allele of interest, but that selection for recovery of the RP elsewhere in the genome also be considered. This two-stage selection can probably be done quite effectively ad hoc by the breeder once the data is adequately summarized; however, Hospital et al. suggest ways to incorporate the two criteria into a selection index such that each component of selection is assured appropriate weighting.

Use of genetic markers can greatly increase the effectiveness of backcrossing, and they should be used in any serious backcrossing program if resources are available to the breeder.

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